

# Nitrogen fixation and transfer in grass-clover leys under organic and conventional cropping systems

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## Abstract

**Background and aim** Symbiotic dinitrogen (N<sub>2</sub>) fixation is the most important external N source in organic systems. Our objective was to compare symbiotic N<sub>2</sub> fixation of clover grown in organically and conventionally cropped grass-clover leys, while taking into account nutrient supply gradients.

**Methods** We studied leys of a 30-year-old field experiment over 2 years in order to compare organic and conventional systems at two fertilization levels. Using <sup>15</sup>N natural abundance methods, we determined the proportion of N derived from the atmosphere (PNdfa), the amount of Ndfa (ANdfa), and the transfer of clover N to grasses for both red clover (*Trifolium pratense* L.) and white clover (*Trifolium repens* L.).

**Results** In all treatments and both years, PNdfa was high (83 to 91 %), indicating that the N<sub>2</sub> fixation

process is not constrained, even not in the strongly nutrient deficient non-fertilized control treatment. Annual ANdfa in harvested clover biomass ranged from 6 to 16 gN m<sup>-2</sup>. At typical fertilizer input levels, lower sward yield in organic than those in conventional treatments had no effect on ANdfa because of organic treatments had greater clover proportions. In two-year-old leys, on average, 51 % of N taken up by grasses was transferred from clover.

**Conclusion** Both, organically and conventionally cropped grass-clover leys profited from symbiotic N<sub>2</sub> fixation, with high PNdfa, and important transfer of clover N to grasses, provided sufficient potassium- and phosphorus-availability to sustain clover biomass production.

**Keywords** <sup>15</sup>N natural abundance · Grass-clover ley · N transfer · Organic cropping · Symbiotic N<sub>2</sub> fixation · *Trifolium* spp.

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## Abbreviations

ANdfa	Amount of nitrogen in clover derived from the atmosphere
BIODYN	Bio-dynamic cropping (with a number 1 or 2 added to indicate low or typical fertilization levels respectively)
BIOORG	Bio-organic cropping (with a number 1 or 2 added to indicate low or typical fertilization levels respectively)
CONFYM	Conventional cropping with mineral fertilization and animal manure (with a number 1 or 2 added to indicate low or typical fertilization levels respectively)

CTRLMIN	Control with mineral fertilization at a typical level
CTRLNON	Non-fertilized control
DOK	Long term field experiment comparing bio-Dynamic bio-Organic and conventional (in German <u>K</u> onventionell) cropping systems
GC	Grass-clover ley (with a number 1 or 2 added to indicate 1- or 2-year-old swards)
PNdfa	Proportion of nitrogen in clover derived from the atmosphere
PNdfc	Proportion of nitrogen in grass derived from clover

## Introduction

Substitution of mineral fertilizer nitrogen (N) by increased exploitation of symbiotic dinitrogen ( $N_2$ ) fixation in agricultural grasslands could be an important contribution to sustainable and resource-efficient agriculture. In organic farming systems symbiotically fixed  $N_2$  is the most important external N source.

In conventionally cropped swards strong benefits of mixing grasses and legumes were achieved in a pan-European experiment over 28 sites in 17 countries (Finn et al. 2013; Kirwan et al. 2007; Lüscher et al. 2008). The contribution of symbiotically-fixed clover N to whole sward N yield can reach  $300 \text{ kgNha}^{-1}\text{yr}^{-1}$  (Nyfeler et al. 2011; Zanetti et al. 1997). These high contributions of symbiotically fixed  $N_2$  were only achieved if two prerequisites were met, namely, high biomass yield of clover, and high proportion of N in clover derived from symbiosis (Nyfeler et al. 2011). However, such studies are rare for organically cropped grass-clover leys (Vinther and Jensen 2000), and implications of the fertilization strategies of organic versus conventional grasslands on symbiotic  $N_2$  fixation have not yet been compared under identical pedo-climatic conditions.

High availability of mineral N from synthetic fertilizers reduces symbiotic  $N_2$  fixation activity of clover and clover proportion in the sward (Boller and Nösberger 1987; Hansen and Vinther 2001; Hebeisen et al. 1997; Nyfeler et al. 2011). Because no synthetic mineral N fertilizers are used in organic farming, and because total N input and input of mineral N (e.g., from slurry) is usually lower in organic than in conventional systems (Dawson et al. 2008), symbiotic  $N_2$  fixation

activity and clover proportions in organic systems might be higher than those found in mineral fertilizer-based systems. However, phosphorus (P) and potassium (K) inputs are also often lower in organic than in conventional cropping systems, resulting in lower plant available P and/or K in the soils under organic cropping (Gosling and Shepherd 2005; Øgaard and Hansen 2010). Limited P and K supply can limit  $N_2$  fixation, as demonstrated by white clover grown in hydroponics, where limited P and K supply restricted  $N_2$  fixation through changes in the relative growth of roots, nodules, and shoots (Hogh-Jensen 2003; Hogh-Jensen et al. 2002). Likewise,  $N_2$  fixation parameters of red clover grown in pots were affected by low P supply as manifested by reduction of nodule number, nodule dry matter, and nitrogenase activity (Hellsten and Huss-Danell 2001). Such down-regulation of symbiotic  $N_2$  fixation seems to be a feedback regulation to adapt symbiosis to the plant's low N requirements induced by strongly limited plant growth (Almeida et al. 2000). These findings suggest that the consistently low P and K balances of organic systems could, over the long term, result in down-regulation of the amount of symbiotically fixed N from the atmosphere. Since symbiosis is the most important external N source of organic systems, this has critical implications for organic farming.

In the DOK (bio-Dynamic, bio-Organic, Konventionell) long term field experiment, organic and conventional cropping systems have been compared at two fertilizer input levels since 1978 (Mäder et al. 2006). In this experiment, P and K inputs and resulting nutrient budgets have been found to be lower in organically than in conventionally cropped soils, resulting in lower P and K in the soils of organic systems as compared to conventional systems (Oberson et al. 2007; Oehl et al. 2002). The DOK experiment provides the opportunity to examine the effects of cropping system-related differences in nutrient supply on ley growth and symbiotic  $N_2$  fixation, because the cropping treatments applied (i) test differences in nutrient supply typical of cropping systems, (ii) test the effects of nutrient supply within the cropping system at two levels, and (iii) strongly extend the range of nutrient scarcity (non-fertilized control). Additionally, (iv) the long-term application of the treatments allows examination of the long-term effects of the applied cropping treatments, including nutrient depletion in the soil.

Symbiotic  $N_2$  fixation can be determined by the  $^{15}\text{N}$  natural abundance method, which is based on the

slight natural differences between the  $^{15}\text{N}$  abundance of soil N and the  $^{15}\text{N}$  abundance of atmospheric  $\text{N}_2$  (Shearer and Kohl 1986; Unkovich et al. 2008). This method has been widely used to estimate symbiotic  $\text{N}_2$  fixation in annual legumes (Douxchamps et al. 2010; Unkovich and Pate 2000) and grasslands (Huss-Danell and Chaia 2005; Jacot et al. 2000; Roscher et al. 2011). In the DOK field experiment, this method has been used to estimate symbiotic  $\text{N}_2$  fixation by soybeans (Oberson et al. 2007), where it revealed a similar proportion of N in soybeans derived from the atmosphere (PNdfa; %) as that found with enriched dilution techniques, and with similar variation. The  $^{15}\text{N}$  natural abundances of non-fixing plants growing alone or in association with a legume have also been used to determine the transfer of N received from an intercropped legume (Daudin and Sierra 2008; Sierra et al. 2007; Snoeck et al. 2000). Sierra et al. (2007) compared the natural abundance method with the  $^{15}\text{N}$  enriched leaf feeding method and found that both methods provide similar estimates of N transfer from the legume to the receiver plant. Based on the long-term DOK experiment, we aimed to gain insight into clover yielding ability, symbiotic  $\text{N}_2$  fixation, and transfer of clover N to the grass as affected by cropping system and nutrient input level. Specifically, we wanted to analyze whether the lower fertilizer N input in organic systems than that of conventional systems, as well as reduced fertilizer input within a given cropping system, would result in higher symbiotic N fixation (expressed as PNdfa and as fixed amounts in  $\text{g N m}^{-2}$ ), or whether this expected increase would be offset by lower P and K supplies. We used the  $^{15}\text{N}$  natural abundance method to determine PNdfa of *Trifolium pratense* L. and *Trifolium repens* L. and determined the N yield of both clover species. The product of these two measures results in the amount of Ndfa (ANdfa;  $\text{g N m}^{-2}$ ), reflecting  $\text{N}_2$  fixation performance of whole sward. The evolution of the  $^{15}\text{N}$  natural abundance in ryegrass over time was used to estimate the transfer of clover N to the grasses.

## Materials and methods

### DOK long term field experiment and its leys

The leys included in this study are located in the DOK long term field experiment located in Therwil near Basel, Switzerland (Mäder et al. 2006). The mean

annual temperature of the site is 9.7 °C and the mean annual precipitation 791 mm (period 1864–2007) (Leifeld et al. 2009). The soil is a Haplic Luvisol developed on deposits of alluvial loess with 15 % sand, 70 % silt, and 15 % clay. The conception and experimental design of the DOK experiment, including a detailed description of the management practices, were presented by Mäder et al. (2002; 2006). Since 1978, three cropping systems have been applied: two organic systems (bio-dynamic = BIODYN; bio-organic = BIOORG) that receive slurry and farmyard manure, and a conventional system (CONFYM) that receives mineral fertilizer, slurry, and farmyard manure. Each of these three systems is managed at two fertilizer input levels: low (level 1) and typical (level 2). Level 2 receives amounts that are typical for the respective cropping system, while level 1 receives half of these amounts. For the organic systems, level 2 is defined by the manure production of 1.4 livestock units (LU), and for the conventional system level 2 is defined by the Swiss fertilization guidelines (Flisch et al. 2009), which recommend moderate input levels compared to other Western European countries. Additionally, a non-fertilized control (CTRLNON) and a control with exclusively mineral fertilizer inputs at level 2 (CTRLMIN) are included, resulting in totally eight treatments. Average annual nutrient inputs to each treatment are presented in Table 1. Forms of nutrient inputs applied are typical for the respective cropping system. The slurries and farmyard manures originate from farms that are managed according to the respective cropping system. Table 1 also shows the average annual N, P, and K budgets for the period from 1978 to 2006, which is calculated as the difference between nutrient input by fertilizers and nutrient output by products removed from the experimental plots. Nutrient status, from 2006, of soils under the different treatments is shown in Table 2.

The DOK experiment has a split-split-plot design in a Latin square with four replicates and a plot size of 5 m×20 m. The seven-year crop rotation is the same for all cropping systems and the same crop rotation is cropped with a time shift on three rotation units (a, b, c) so that three of the seven crops are present each year for each cropping system. Crop rotation includes silage maize (*Zea mays* L.), winter wheat I (*Triticum aestivum* L.), soybeans [*Glycine max* (L.) Merr.], potatoes (*Solanum tuberosum* L.), winter wheat II, and two subsequent years of grass-clover (GC) ley. Leys

**Table 1** Fertilization and plant protection in the organic and conventional cropping systems and in the unfertilized and mineral fertilized controls of the long term field experiment, with average N, P, and K inputs and balances for 29 years (1978–2006)

Characteristics	Cropping systems and control treatments					Minerally fertilized control (CTRLMIN) <sup>a</sup>
	Unfertilized control (CTRLNON)	Bio-dynamic (BIODYN)	Bio-organic (BIOORG)	Conventional-manure (CONFYM)		
Fertilization						
Fertilizer input level	zero	1=low	2=typical	1=low	2=typical	2=typical
Type of fertilizer	Non-fertilized control	Aerobically composted farmyard manure (FYM) and slurry <sup>b</sup>	Slightly aerobically rotted FYM and slurry <sup>b</sup>	Stacked FYM and slurry and mineral fertilizer as supplement	Minerally fertilized control	
Manure LU ha <sup>-1</sup> yr <sup>-1</sup>	0	0.7 <sup>c</sup>	1.4 <sup>c</sup>	0.7 <sup>c</sup>	1.4 <sup>c</sup>	0
Mineral fertilizer level	0	0	0	Completed to half	full norm <sup>d</sup>	Norm <sup>d</sup>
Total N input (kg ha <sup>-1</sup> yr <sup>-1</sup> )	0	49	99	53	107	122
Mineral N input (kg ha <sup>-1</sup> yr <sup>-1</sup> )	0	16	32	18	37	122
N balance (kg ha <sup>-1</sup> yr <sup>-1</sup> ) <sup>e</sup>	-144	-140	-106	-140	-100	-110
Total P input (kg ha <sup>-1</sup> yr <sup>-1</sup> )	0	12	23	14	27	39
P balance (kg ha <sup>-1</sup> yr <sup>-1</sup> ) <sup>e</sup>	-19	-15	-8	-15	-5	5
Total K input (kg ha <sup>-1</sup> yr <sup>-1</sup> )	0	82	163	82	164	249
K balance (kg ha <sup>-1</sup> yr <sup>-1</sup> ) <sup>e</sup>	-75	-50	-13	-53	-17	14
Plant protection						
Weed control	Mechanical					Mechanical and herbicides
Disease control	Indirect methods			Indirect methods, until 1991 copper		Fungicides (thresholds)
Insect control	Plant extracts, bio-control					Insecticides (thresholds)
Special treatments	Biodynamic preparations <sup>f</sup>			None		Plant growth regulators

<sup>a</sup> CTRLMIN was from 1978 to 1985 non-fertilized. The values displayed are the averages over the years 1985–2006

<sup>b</sup> In the early phase of this experiment, some rock phosphate was applied to BIODYN and rockdust to BIOORG

<sup>c</sup> Before 1992, 0.6 and 1.2 livestock units (LU) ha<sup>-1</sup> yr<sup>-1</sup>, respectively

<sup>d</sup> Flisch et al. (2009)

<sup>e</sup> Difference between total N, P, or K input by fertilizers and N, P, or K output by products removed at harvest

<sup>f</sup> Biodynamic preparations to strengthen plant resistance to pests and diseases, and for the promotion of plant growth and development (Mäder et al. 2002)

**Table 2** Nutrient status of the soils in the top layer (0–20 cm) sampled in 2006

Treatment		pH <sup>b</sup>	Total C <sup>c</sup>	Total N <sup>c</sup>	Available P <sup>d</sup>	Available K <sup>d</sup>
CropSyst <sup>a</sup>	FertLevel <sup>a</sup>		g kg <sup>-1</sup>	g kg <sup>-1</sup>	mg kg <sup>-1</sup>	mg kg <sup>-1</sup>
CTRLNON	0	5.8 a	9.9 a	1.4 a	0.22 a	2.4 a
BIODYN	1	6.4 cd	12.1 bcd	1.6 bc	0.43 b	4.1 ab
BIODYN	2	6.7 e	14.2 e	1.8 d	0.84 c	8.3 c
BIOORG	1	6.1 b	11.4 bc	1.5 ab	0.43 b	4.6 b
BIOORG	2	6.4 cd	12.6 d	1.6 dc	0.84 c	10.2 d
CONFYM	1	6.1 b	10.9 ab	1.4 ab	0.53 b	4.2 ab
CONFYM	2	6.3 bc	12.2 dc	1.6 bc	1.43 d	10.1 cd
CTRLMIN	2	6.6 de	11.3 bc	1.4 ab	0.92 c	9.1 cd
SEM		0.08	0.44	0.06	0.06	0.6
Anova source of variation with (DF)						
Treatments <sup>c</sup>	(7)	***	***	***	***	***
CropSyst (S) <sup>f</sup>	(2)	***	**	**	***	x
FertLev (F) <sup>f</sup>	(1)	***	***	***	***	***
S × F <sup>f</sup>	(2)	n.s.	n.s.	n.s.	***	n.s.

Average of 8 replicate plots of rotation units c and b; <sup>x</sup>, \*, \*\*, \*\*\* significant at  $p < 0.1$ , 0.05, 0.01 and 0.001 probability level, respectively; *n.s.* not significant; *SEM* standard error of the mean; *DF* degrees of freedom; values followed by the same letter are not significantly different (Fisher's LSD,  $p < 0.05$ )

<sup>a</sup> For cropping systems (CropSyst) and fertilizer input level (FertLev) see Table 1

<sup>b</sup> pH determined in water

<sup>c</sup> NCS elemental analyser (Flash EA 1112 Series NCS analyser, Thermo Fisher Scientific, Waltham, MA, USA)

<sup>d</sup> Determined using CO<sub>2</sub>-saturated water extraction of Dirks and Scheffer (1930)

<sup>e</sup> Treatment denotes analysis of variance over all eight treatments

<sup>f</sup> Two way Anova by cropping system and fertilization level including the systems BIODYN, BIOORG, CONFYM at fertilizer input levels 1 and 2

are sown in August after the harvest of winter wheat, soil preparation by plowing, and harrowing, and basic fertilizer application. Leys are used for two full vegetation periods (GC1, GC2). In the spring of the third vegetation period, swards are broken up by plowing prior to seeding the successive silage maize crop.

All GC leys between 2007 and 2008 were used in this study. In 2007, GC1 was growing on rotation unit b and GC2 on unit c. In 2008 the GC growing on unit b went into the 2nd year of utilization. The GC leys contained the following species: white clover (*Trifolium repens* L.), red clover (*Trifolium pratense* L.), perennial ryegrass (*Lolium perenne* L.), cocksfoot (*Dactylis glomerata* L.), meadow fescue (*Festuca pratensis* Huds.) and timothy (*Phleum pratense* L.). Leys are harvested five times per vegetation period, with the first harvest early in May and the last harvest late in October. The timing of fertilization and the fertilizer rates applied

at level 2 to the leys are presented in Table 3. Timing, forms, and amounts of fertilizer inputs to leys were identical in 2007 and 2008.

#### Sampling of the leys

Table 4 presents an overview of the sampling and analyses done on plant samples collected from 2007 to 2009 for this study. Emphasis was placed on the GC2 sward growing in 2007 (unit c) because we expected that in two-year-old leys any treatment effects would be most recognizable. Therefore, in 2007, each of the eight treatments in unit c (i.e., 32 plots in total) was sampled at each of the five harvests. Additionally, in order to compare symbiotic N<sub>2</sub> fixation within the same calendar year in one- and two-year-old swards, we also sampled the CTRLNON and all fertilizer input level 2 treatments (i.e., 20 plots in

**Table 3** Nitrogen, P and K fertilization at the typical fertilizer input level in grass-clover leys, and  $^{15}\text{N}$  isotopic signature of the N fertilizers

Cropping system <sup>a</sup>	Fertilizer	Applications <sup>c</sup>		Total N <sup>d</sup> kg ha <sup>-1</sup> yr <sup>-1</sup>	Mineral N <sup>de</sup> kg ha <sup>-1</sup> yr <sup>-1</sup>	$\delta^{15}\text{N}^f$ ‰	Total P <sup>d</sup> kg ha <sup>-1</sup> yr <sup>-1</sup>	Total K <sup>d</sup>
		Timing	Number					
BIODYN	Farmyard manure <sup>b</sup> , Slurry	Start of vegetation, after harvest 1, 2	3	51	17	15 (1.8)	28	137
BIOORG	Farmyard manure <sup>b</sup> ; Slurry	Start of vegetation, after harvest 1	2	47	13	10 (1.7)	12	120
CONFYM	Farmyard manure <sup>b</sup> , Slurry	Start of vegetation, after harvest 1, 2, 3	4	180	131	4 (0.8)	35	396
CTRLMIN	Calcium-Ammonium-Nitrate	Start of vegetation, after harvest 1, 2, 3	4	150	150	-0.7 (0.02)	40	314

<sup>a</sup>For cropping systems see Table 1

<sup>b</sup>Farmyard manure only applied at sowing of the leys

<sup>c</sup>Same for leys in first and second year of utilization

<sup>d</sup>Dose applied at the typical fertilization level 2; half of this dose was applied at the low fertilization level of BIODYN, BIOORG and CONFYM

<sup>e</sup>Ammonium-N in animal manure

<sup>f</sup>Average and SEM of 8, 6, 10, and 6 samples for BIODYN, BIOORG, CONFYM, and CTRLMIN, respectively

total) in the GC1 sward in 2007. To compare symbiotic  $\text{N}_2$  fixation of the two-year-old leys across two calendar years, we sampled all eight treatments of the first and third harvest from the GC2 sward growing in 2008. The sampling done in 2008 was also important for following the time course of the  $^{15}\text{N}$  natural abundances over 2 years within the same plots. This monitoring was continued into 2009 when samples were taken in April before the swards were broken up by plowing.

During each sampling, all four field replicates per treatment were sampled and samples separated to determine yield proportion of the botanical fractions white clover, red clover, and grasses in the sward and to get plant material of individual botanical fractions for chemical analyses. An area of 0.5 m × 0.5 m with 1 m distance to the plot border was cut 4 cm above the ground using electric scissors. The harvested plant material was kept at 4 °C until it was separated into the botanical fractions (within 2 days

**Table 4** Overview of plant sampling and analyses by harvests, where harvest number 1 = first and 5 = last harvest per year. Harvested material was separated into grass, white clover, and red clover, and analyses were conducted on each fraction

Year	Rotation unit	Age of ley years	Treatments	Harvests analyzed/considered for					
				Dry matter	Clover proportion <sup>a</sup>	N concentration	$\delta^{15}\text{N}$ , PNdfa	Other elements	ANdfa
2007	C	2 (GC2)	All	All (1 to 5)	All (1 to 5)	All (1 to 5)	1, 3	1,3	All (1 to 5)
2007	B	1 (GC1)	CTRLNON and all level 2	All (1 to 5)	All (1 to 5)	All (1 to 5)	1, 3	1,3	All (1 to 5)
2008	B	2 (GC2)	All	All (1 to 5) <sup>b</sup>	1, 3	1, 3	1, 3	1,3	All <sup>c</sup>
2009 <sup>d</sup>	B	3	CTRLNON and all level 2	1 <sup>d</sup>		1 <sup>d</sup>	1 <sup>d</sup>	none	1 <sup>d</sup>

<sup>a</sup>Proportion of red and white clover in total dry matter

<sup>b</sup>Harvest 2, 4, and 5 were not separated into botanical fractions

<sup>c</sup>Annual ANdfa determined using average clover proportion from harvests 1 and 3

<sup>d</sup>Early harvest at reduced biomass prior to destruction of ley by plowing



after sampling). From the grass fraction, a sub-sample of ryegrass, which was usually the dominating grass, was taken to be used as a reference plant to determine N derived from symbiotic N<sub>2</sub> fixation (see details below). Botanical fractions were dried at 60 °C for 3 days and their dry matter (DM) weight was used to calculate their proportion in the sward. This was done at each sampling, i.e., for all five harvests of GC1 and GC2 in 2007, and for harvest 1 and 3 of GC2 in 2008. All sampled fractions from each sampling were analysed for N concentration. Additionally, red clover, white clover, and ryegrass sampled at harvest 1 and 3 in 2007 and 2008 were analysed for total P and K concentrations and  $\delta^{15}\text{N}$ .

The day after sub-plot sampling, yield determination of the whole plots was conducted over an area of 1.5 m × 10 m with a plot harvester (Hege 212). The fresh weight of the plant biomass was weighed by the harvester automatically in the field, and a subsample was taken for DM determination in order to derive DM yield. The DM yields were determined for all treatments and all harvests for all studied leys. The DM yields of the botanical fractions were obtained by multiplying their proportion (obtained from the sub-plot sampling) with the DM yield of the whole plot. These DM yields were used to calculate N yield of the fractions and amounts of N fixed by red and white clover.

#### Clover N derived from atmosphere

At the first and third harvest of both years, the proportions of N derived from the atmosphere (PNdfa, in %) in white and red clover were assessed using the <sup>15</sup>N natural abundance method (Shearer and Kohl 1986).

The <sup>15</sup>N abundance values are expressed as  $\delta^{15}\text{N}$ , i.e., per mil (‰) <sup>15</sup>N excess (or depletion) over the <sup>15</sup>N abundance of the atmosphere (= 0.36637 atom% <sup>15</sup>N) (Shearer and Kohl 1986):

$$\delta^{15}\text{N}(\text{‰}) = \frac{\text{atom}\% \text{ } ^{15}\text{N sample} - \text{atom}\% \text{ } ^{15}\text{N air}}{\text{atom}\% \text{ } ^{15}\text{N air}} \times 1000 \quad (1)$$

The PNdfa (in %) is (Shearer and Kohl 1986):

$$\text{PNdfa}(\%) = \frac{\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{clover}}}{\delta^{15}\text{N}_{\text{ref}} - B} \times 100 \quad (2)$$

where  $\delta^{15}\text{N}_{\text{ref}}$  is the  $\delta^{15}\text{N}$  of a non-fixing reference plant (whole shoot);  $\delta^{15}\text{N}_{\text{clover}}$  is the  $\delta^{15}\text{N}$  of white clover or red clover (whole shoot);  $B$  is the  $\delta^{15}\text{N}$  of

white clover or red clover shoots relying on atmospheric N<sub>2</sub> as a sole source of N and accounts for any internal isotopic fractionation of legume plants (Unkovich et al. 1994).

For the reference plant, we used perennial ryegrass growing in the sampling area, i.e., in association with the clover so that the  $\delta^{15}\text{N}$  of non N<sub>2</sub>-fixing reference plants is identical to the  $\delta^{15}\text{N}$  of soil N utilised by the legume (Unkovich et al. 2008). In the case of the fertilized treatments, mineral N taken up is composed of mineralized soil N and fertilizer N. Preliminary testing showed that the  $\delta^{15}\text{N}$  of the ryegrass fraction did not differ from  $\delta^{15}\text{N}$  of cocksfoot or the whole grass fraction, and ryegrass usually constituted most of the grass fraction.

As B values we used the lowest detected  $\delta^{15}\text{N}$  in the field (Carlsson et al. 2009; Hansen and Vinther 2001; Roscher et al. 2011). As  $\delta^{15}\text{N}$  in clover was not significantly affected by treatment and not significantly changed over time (see below) we chose the lowest value from all treatments in years 2007 and 2008, resulting in B values of -1.0‰ ( $n=16$ , standard deviation (SD)=0.2‰) for red clover and -1.1‰ ( $n=16$ , SD=0.2‰) for white clover. We used the same B values for both years of the study because Carlsson et al. (2006) found only a very small change in B value after a simulated winter. Our B values were similar to B values reported earlier for *T. repens* and *T. pratense* (Carlsson et al. 2006; Huss-Danell and Chaia 2005; Roscher et al. 2011).

The amount of N fixed per m<sup>2</sup> was calculated for each legume species as:

$$\text{ANdfa}(\text{g N m}^{-2}) = \text{clover N yield}(\text{g m}^{-2}) \times 0.01 \times \text{PNdfa}(\%) \quad (3)$$

Where

$$\text{Clover N yield}(\text{g m}^{-2}) = \text{clover DM}(\text{kg m}^{-2}) \times \text{shoot N concentration}(\text{g N kg}^{-1}\text{DM}). \quad (4)$$

ANdfa of both species was totaled to derive ANdfa per harvest, and annual ANdfa was calculated as the sum of ANdfa of the five harvests. To calculate ANdfa of harvests 2, 4, and 5, we used the average PNdfa of harvests 1 and 3 for each clover type and treatment combination, which was then multiplied with the respective clover N yields determined for harvests 2, 4, and 5.

Because PNdfc changed little with time (see [Results](#) section), this provided reasonable estimates of ANdfc.

#### Grass N derived from clover

The proportion of N in the grasses derived from clover (PNdfc, in %) was calculated from the changes of  $\delta^{15}\text{N}$  over time by adapting the formula of Daudin and Sierra (2008):

$$\text{PNdfc}(\%) = \frac{\delta^{15}\text{N}_{\text{grass-t0}} - \delta^{15}\text{N}_{\text{grass-tj}}}{\delta^{15}\text{N}_{\text{grass-t0}} - \delta^{15}\text{N}_{\text{clover-tj}}} \times 100 \quad (5)$$

Where  $\delta^{15}\text{N}_{\text{grass-t0}}$  is the  $\delta^{15}\text{N}$  of ryegrass at time zero (start), which was at the first harvest of GC1 in 2007, assuming that at this initial harvest ryegrass had not yet taken up clover derived N and, thus, its  $\delta^{15}\text{N}$  reflects the isotopic composition of available mineral soil and fertilizer N of the respective system;  $\delta^{15}\text{N}_{\text{grass-tj}}$  is the  $\delta^{15}\text{N}$  of ryegrass of later harvests (harvest 3 in 2007; harvests 1 and 3 in 2008, harvest 1 in 2009);  $\delta^{15}\text{N}_{\text{clover-tj}}$  is the  $\delta^{15}\text{N}$  of clover at the same later harvests, i.e., the  $\delta^{15}\text{N}$  of the legume N source that can be transferred to grass.

#### Sample preparation and analyses

Samples were milled (particle size  $\sim 1$  mm) using a cutting mill (Retsch GmbH, Germany). Subsequently, we pulverized a subsample using a ball mill (Retsch GmbH, Germany). For P and K analysis, we incinerated milled samples at 550 °C for 8 h and solubilized the ashes in 15 M nitric acid at room temperature. The P and K concentrations were measured with an ICP-MS (Agilent, USA). The N concentration was measured by dry combustion with a NCS elemental analyser (Flash EA 1112 Series NCS analyser, Thermo Fisher Scientific, Waltham, MA, USA). Total N concentration and atom%  $^{15}\text{N}$  were analysed on a continuous flow ANCA-NT gas/solid/liquid preparation module coupled to a tracers mass spectrometer (Europa Scientific, England; precision  $\pm 0.2\delta$  per mil).

#### Statistical analyses

Statistical analyses were carried out using the Linear Mixed Models procedure in the statistical analysis package SYSTAT 12 (Systat Software Inc., Chicago, USA). For analysis of variance, proportions were

transformed using arcsin-transformation. Testing of the treatment effect included all treatments, and the standard error of the mean (SEM) was derived from this analysis. The effect of cropping system (S), fertilization level (F), and the interaction of these two factors was tested using fertilizer levels 1 and 2 for BIODYN, BIOORG, and CONFYM. Pairwise comparisons were carried out by Fisher's least significant difference or *t*-test at  $p=0.05$ .

## Results

### Dry matter production, clover proportion and clover yield

The total annual DM production of the two-year-old grass-clover leys was between 434 and 1,322  $\text{gm}^{-2}$  (Table 5). Yield of GC2 was significantly lower in 2008 than in 2007 ( $p<0.001$ ). Yields of GC1 and GC2 growing in 2007 were not significantly different, i.e., there was no significant effect of sward age (results not shown). Most importantly, yields were always significantly affected by the treatments (Table 5). Firstly, organically cropped leys produced 84 to 87 % of the yields of those from conventional cropping when compared at typical fertilizer level, and plots left unfertilized since 1978 produced 42 %. Secondly, dry matter yield was higher with typical than with low fertilizer input levels for each cropping system. These treatment effects were stable for both years and both sward ages (interactions treatment  $\times$  year and treatment  $\times$  age of sward not significant). The monitoring of all individual harvests from 2007 to spring 2009 in selected treatments showed that treatment effects were even largely maintained at the single harvest scale (data not shown).

In the GC2 sward growing in 2007, red and white clover together contributed 29 to 53 % of total harvested biomass (Table 5). The clover proportion was significantly affected by the treatments: the two organic systems (BIODYN and BIOORG) and the unfertilized plots had higher clover proportions than CONFYM and CTRLMIN. A similar pattern was shown for the GC1 sward growing in 2007 and the GC2 sward growing in 2008. Interactions between treatments and years, or between treatments and age of sward, were not significant. Reduced fertilization tended to result in higher clover proportions. The



monitoring of the individual harvests from 2007 to spring 2009 showed that treatment effects were largely maintained at the single harvests even though clover proportion fluctuated with time (data not shown).

In 2007, the clover fraction of GC2 was dominated by red clover (on average 74 % of clover N yield, Table 6), while white clover dominated clover biomass of GC1 in 2007 (58 % of clover N yield), and GC2 in 2008 (66 % of clover N yield). The proportion of red clover in the clover biomass significantly fluctuated over time but was not significantly affected by the treatment (data not shown). Annual clover yield (sum of red and white clover) was less clearly affected by treatments than the total DM yield (Table 5), but clover yield was always lowest in CTRLNON. The significant treatment effects on total DM production (Table 5) were, therefore, largely due to significant differences in grass yields (Table 6).

#### Nutrient concentrations in clover and grass

Nitrogen concentration in red and white clover was for each treatment higher than  $31 \text{ mg g}^{-1}$ , and was higher than in ryegrass, where it ranged from 22 to  $26 \text{ mg g}^{-1}$  (Table 7). The N concentrations in both clovers and in ryegrass were not significantly affected by the treatments. The N concentrations in ryegrass were not significantly different from the N concentration of the whole grass fraction. The P concentrations were lower in clover (2.2 to  $3 \text{ mg g}^{-1}$ ) than in ryegrass (2.7 to  $4.4 \text{ mg g}^{-1}$ ). Potassium concentrations in both clover species and ryegrass varied broadly, from 6 to  $37 \text{ mg g}^{-1}$ . The concentrations of P and K were significantly lower with the low than typical fertilization level, with the most pronounced effects observed for K. Treatment effects on P and K concentrations were the same for all studied leys, and there were no significant interactions between treatments and year, and treatment and age of sward.

#### $\delta^{15}\text{N}$ isotopic signatures and clover N derived from atmosphere

The monitoring of  $\delta^{15}\text{N}$  from May 2007 until April 2009 showed that  $\delta^{15}\text{N}$  in red and white clovers was already below 0 in the first year of utilization of the grass-clover ley and remained low over the duration of this study (Fig. 1). The  $\delta^{15}\text{N}$  of both clovers were not significantly affected by the treatments. In contrast, the  $\delta^{15}\text{N}$  of ryegrass was significantly affected by

treatments (Fig. 1,  $p < 0.05$ ) and significantly decreased with time, particularly from year 1 to year 2 ( $p < 0.001$ ). Throughout the study, the  $\delta^{15}\text{N}$  values of the ryegrass were significantly higher than the  $\delta^{15}\text{N}$  values of both clovers ( $p < 0.001$ ) (Fig. 1).

The clear and significant differences in  $\delta^{15}\text{N}$  between clover and ryegrass translated into high PNdfa, reaching between 83 % and 89 % for red clover and between 85 % and 91 % for white clover across all eight treatments studied in the GC2 sward growing in 2007 (Table 6). Likewise, the PNdfa for red clover and white clover monitored from 2007 until early 2009 was, on average, 90 % and 91 %, respectively (for all treatments and harvests, Fig. 2). The PNdfa was not significantly affected by the treatment or clover species and remained at high levels, although the decrease over time was significant ( $p < 0.05$ ).

The annual amounts of N fixed in the biomass of red and white clover (sum of five harvests) ranged from 6 to  $16 \text{ g m}^{-2}$  (Table 6). The ANdfa was not significantly affected by treatments, but was lowest in CTRLNON and tended to be highest in the organic systems with typical fertilizer input levels.

#### Grass N derived from clover

The decrease of  $\delta^{15}\text{N}$  in ryegrass with time suggested that 46 to 60 % of N in the ryegrass growing in the two-year-old leys originated from clover N (Fig. 3). Assuming that grasses of the GC2 sward growing in 2007, for which total N uptake by the grasses was determined for all five harvests (Table 6), would have received the same proportion of Ndfc as that in the GC2 sward growing in 2008, this would correspond to 4 to  $11 \text{ g N m}^{-2}$  of grass N yield derived from clover. On average, 87 % of clover N was derived from the atmosphere (Table 6), which indicates that for grass N yield an additional amount of atmospheric N of 3.5 to  $10 \text{ g N m}^{-2}$  was harvested. Because the proportion of Ndfc was similar in all treatments, the amount of clover N in grasses was higher with greater grass N yields.

## Discussion

#### Nutrient status and dry matter yield

Dry matter yields obtained with CTRLMIN and CONFYM at level 2 were at the same yield level

**Table 5** Total annual dry matter yield (sum of five harvests) and clover proportion of total dry matter of two-year-old grass-clover meadows (GC2) growing in 2007 and 2008 under

organic and conventional cropping systems with low (1) or typical (2) fertilizer input levels

Treatment <sup>a</sup>		2007		2008			
CropSyst <sup>a</sup>	Fertlevel <sup>a</sup>	Total yield g DM m <sup>-2</sup>	Clover. proportion % of DM	Clover yield g DM m <sup>-2</sup>	Total yield g DM m <sup>-2</sup>	Clover. proportion <sup>b</sup> % of DM	Clover yield <sup>c</sup> g DM m <sup>-2</sup>
CTRLNON	0	651 a	51 c	338	434 a	43	189
BIODYN	1	1021 bc	49 c	503	676 b	40	270
BIODYN	2	1061 bc	46 bc	487	1001 cd	41	413
BIOORG	1	981 b	51 c	495	827 bc	47	391
BIOORG	2	1163 cd	53 c	618	992 cd	38	410
CONFYM	1	1245 d	39 abc	496	943 cd	33	323
CONFYM	2	1322 d	28 a	383	1139 de	25	311
CTRLMIN	2	1257 d	29 ab	372	1226 e	30	365
SEM		59	5	80	77	6	77
Anova source of variation with (DF)							
Treatment <sup>c</sup>	(7)	***	*	n.s.	***	n.s.	n.s.
CropSyst (S) <sup>d</sup>	(2)	**	**	n.s.	x	x	n.s.
FertLev (F) <sup>d</sup>	(1)	x	n.s.	n.s.	**	n.s.	n.s.
S × F <sup>d</sup>	(2)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

<sup>x</sup>, <sup>\*</sup>, <sup>\*\*</sup>, <sup>\*\*\*</sup> significant at  $p < 0.1$ , 0.05, 0.01 and 0.001 probability level, respectively; *n.s.* not significant; *SEM* standard error of the mean; *DF* degrees of freedom; values followed by the same letter are not significantly different (Fisher's LSD,  $p < 0.05$ )

<sup>a</sup> For cropping systems (CropSyst) and fertilizer input level (FertLev) see Table 1

<sup>b</sup> Weighted mean of clover proportion determined for harvests 1 and 3 only in 2008; in 2007 clover proportion was determined for each of the five harvests

<sup>c</sup> Treatment denotes analysis over all eight treatments

<sup>d</sup> Two way Anova by cropping system and fertilization level including systems BIODYN, BIOORG, and CONFYM at fertilizer input levels 1 and 2

reported for intensively managed grassland under farming conditions in Switzerland (Flisch et al. 2009), but were somewhat lower than those of other small plot experiments (Hebeisen et al. 1997; Nyfeler et al. 2009). We suggest that this is related to the relatively low precipitation at the site where the DOK experiment is located. Reduced yields under organic cropping agree with Mäder et al. (2006) and Gunst et al. (2007), who reported after 27 years of DOK field experimentation that grass-clover leys of organic systems reached, on average, 87 % of the yields of CONFYM. Reduced fertilization (level 1 vs. level 2) significantly reduced yields of organic systems while the reduction was less pronounced in the conventional system, as reported by Jossi et al. (2009) for ley yields of the DOK experiment from 1992 to 2005. This is because CONFYM level 1 received nutrient inputs similar to organic treatments

at typical levels (Tables 1 and 3). We sampled the GC2 sward growing in 2007 intensively and consider it representative because of the yield level and because there were no interactions between treatments and years. The total DM yields were significantly positively correlated to the K and P concentrations in plants, to the mineral N input, and to available soil P, while correlations with N concentrations in grass and clover were not significant (Table 8).

Different P and K balances induced by the treatments of the field experiment (Table 1) resulted in a gradient of available P and K in the soil (Table 2), and P and K concentrations in the plant biomass (Table 7), as reported in earlier studies (Oberson et al. 2007; Oehl et al. 2002). The P and K concentrations in the clover and grass shoot biomass were significantly correlated with soil available P and K (Table 8). Application of the interpretation scheme of Flisch et

**Table 6** Annual nitrogen (N) yield of red and white clovers and grass, proportion (PNdfa) of N in red and white clovers derived from the atmosphere, and annual amount of N in clover derived from the atmosphere (ANDfa) in two-year-old grass-clover meadows (GC2) growing under organic or conventional cropping systems with low (1) or typical (2) fertilizer input levels in 2007 and 2008

Treatment		2007					2008		
CropSyst <sup>a</sup>	FertLev <sup>a</sup>	White clover N yield g m <sup>-2</sup>	White clover PNdfa <sup>b</sup> %	Red clover N yield g m <sup>-2</sup>	Red clover PNdfa <sup>b</sup> %	Clover ANDfa <sup>c</sup> g m <sup>-2</sup>	Grass N yield g m <sup>-2</sup>	Clover ANDfa <sup>c</sup> g m <sup>-2</sup>	
CTRLNON	0	4.4	91	6.9	89	10.0	6.5 a	6.2	
BIODYN	1	4.8	91	12.0	87	14.7	11.6 b	8.7	
BIODYN	2	2.4	90	12.6	86	12.8	13.6 b	13.2	
BIOORG	1	4.1	91	12.1	86	14.2	11.2 b	12.9	
BIOORG	2	4.3	91	14.8	83	16.1	13.3 b	13.7	
CONFYM	1	4.7	87	12.0	84	14.0	17.6 c	10.7	
CONFYM	2	3.3	86	8.9	87	10.4	21.9 d	9.2	
CTRLMIN	2	3.0	85	9.1	84	9.9	22.0 d	11.4	
SEM		1.1	3.4	2.3	2.5	2.1	1.3	2.4	
Anova Source of variation with (DF)									
Treatment <sup>d</sup>	(7)	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	
CropSyst (S) <sup>e</sup>	(2)	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	
FertLev (F) <sup>e</sup>	(1)	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	
S × F <sup>e</sup>	(1)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

<sup>x</sup>, \*, \*\*, \*\*\* significant at  $p < 0.10$ , 0.05, 0.01 and 0.001 probability level, respectively; *n.s.* not significant; *SEM* standard error of the mean; *DF* degrees of freedom; values followed by the same letter are not significantly different (Fisher's LSD,  $p < 0.05$ )

<sup>a</sup> For cropping systems (CropSyst) and fertilizer input level (FertLev) see Table 1

<sup>b</sup> Average of PNdfa determined for harvests one and three

<sup>c</sup> Sum of N fixed by all clover (red and white) in five harvests

<sup>d</sup> Treatment denotes analysis over all eight treatments

<sup>e</sup> Two way Anova by cropping system and fertilization level, including systems BIODYN, BIOORG, and CONFYM at fertilizer input levels 1 and 2

al. (2009) suggests that soils of CTRLNON and treatments with low fertilization levels had low and moderate available soil P, respectively, while soils under treatments with typical fertilization had sufficient available P. Available soil K was classified as low for CTRLNON and all low fertilization treatments and as moderate for treatments with typical fertilization.

The interpretation of nutrient concentrations in grasslands is difficult because of nutrient interactions and because nutrient concentrations are lower with greater DM production (Duru and Ducrocq 1997; Jouany et al. 2004). For field grown perennial ryegrass, Bailey et al. (1997b) compiled from various previous studies the following critical concentrations under which the given nutrient would limit ryegrass

yield: 28, 2.5 and 20 mg g<sup>-1</sup> for N, P, and K, respectively. For ryegrass grown under the controlled conditions of a fertilizer experiment, Bailey et al. (1997a) proposed norm ratios of 9.0, 1.2 and 8.5 for N:P, N:K, and K:P, respectively. Liebisch et al. (2013) suggested for grasses growing in fields under a range of use intensities, N:P ratios from 5.5 to 9 and K:P from 6 to 10.5 are optimal, but also concluded that nutrient ratios are not reliable in defining the plant nutrient status in case of co-limitation. In the present study, the K concentration of 11 mg g<sup>-1</sup> and the average K:P ratio of 4 indicate that K strongly limited grass yield in CTRLNON. Average K:P ratios of 6.6, 7.5, and 7.6 in BIOORG level 1, BIODYN level 1 and BIOORG level 2, respectively, indicate K limitation also existed

**Table 7** Nitrogen, P and K concentrations in red clover, white clover, and ryegrass of two-year-old grass-clover leys sampled in 2007 under organic and conventional cropping

Treatment		Red clover			White clover			Ryegrass		
CropSyst	FertLev	N <sup>b</sup> mg g <sup>-1</sup>	P <sup>c</sup>	K <sup>c</sup>	N <sup>b</sup> mg g <sup>-1</sup>	P <sup>c</sup>	K <sup>c</sup>	N <sup>b</sup> mg g <sup>-1</sup>	P <sup>c</sup>	K <sup>c</sup>
CTRLNON	0	32.0	2.2 a	6.5 a	37.1	2.3 ab	7.4 a	22.3	2.7 a	10.9 a
BIODYN	1	33.6	2.2 a	11.8 b	36.1	2.2 a	15.0 b	22.4	2.8 ab	21.1 b
BIODYN	2	31.3	2.5 b	17.2 c	36.7	2.8 c	23.7 c	24.4	3.7 cd	31.0 de
BIOORG	1	32.6	2.3 ab	11.5 b	37.4	2.3 ab	14.3 b	23.7	3.9 cde	25.9 c
BIOORG	2	32.1	2.7 c	15.3 bc	37.3	3.1 c	21.5 c	24.9	4.1 de	31.2 de
CONFYM	1	32.7	2.5 b	15.8 c	35.8	2.7 bc	21.4 c	24.6	3.3 bc	28.4 cd
CONFYM	2	32.7	2.8 c	26.9 d	36.1	2.8 c	31.7 d	23.9	4.0 de	35.2 ef
CTRLMIN	2	33.5	2.8 c	28.8 d	37.0	3.0 c	30.7d	25.5	4.4 c	37.2 f
SEM		0.8	0.1	1.4	0.6	0.2	1.7	0.9	0.2	2.4
Anova Source of variation with (DF)										
Treatments <sup>d</sup>	(7)	n.s.	***	***	n.s.	**	***	x	***	***
CropSyst(S) <sup>e</sup>	(2)	n.s.	*	***	n.s.	n.s.	***	n.s.	**	**
FertLev(F) <sup>e</sup>	(1)	n.s.	***	***	n.s.	***	***	n.s.	***	***
S × F <sup>e</sup>	(2)	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

x, \*, \*\*, \*\*\* significant at  $p < 0.10, 0.05, 0.01$  and  $0.001$  probability level, respectively; *n.s.* not significant; *SEM* standard error of the mean; *DF* degrees of freedom; means followed by the same letter are not significantly different (Fisher's LSD,  $p < 0.05$ )

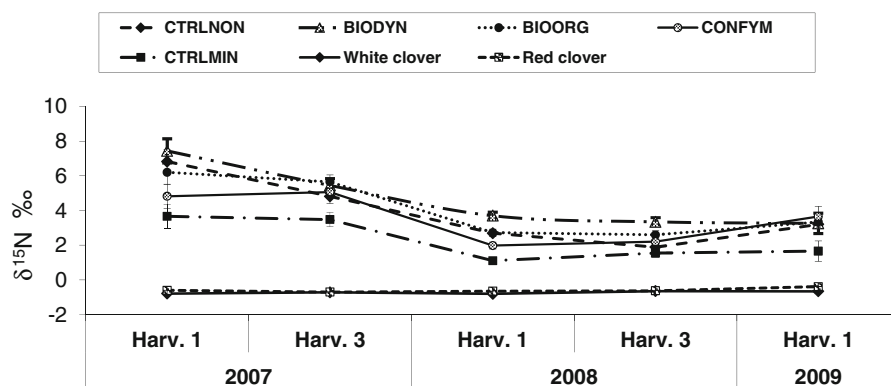
<sup>a</sup> For cropping systems (CropSyst) and fertilizer input level (FertLev) see Table 1

<sup>b</sup> Average of concentrations measured for all five harvests

<sup>c</sup> Average of concentrations measured for harvests 1 and 3

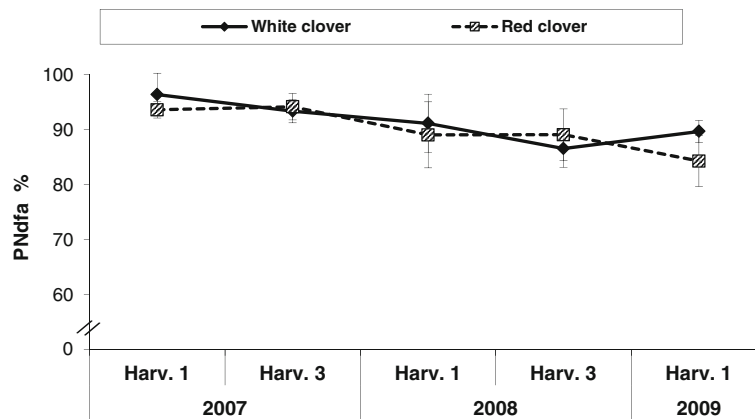
<sup>d</sup> Treatment denotes analysis over all eight treatments

<sup>e</sup> Two way Anova by cropping system and fertilization level including systems BIODYN, BIOORG, and CONFYM at fertilizer input levels 1 and 2



**Fig. 1** Evolution of  $\delta^{15}\text{N}$  (‰) over time in ryegrass and clover sampled in grass-clover leys under organic (BIODYN, BIOORG) and conventional (CONFYM) cropping systems with typical fertilizer input levels, in the unfertilized control (CTRLNON), and in the mineral fertilized control (CTRLMIN). 2007 was the first

(GC1), 2008 the second (GC2), and 2009 the beginning of the third year of ley growth. Because the  $\delta^{15}\text{N}$  for clover was not significantly affected by the treatments, average and standard error of mean over all five treatments is shown for *white* and *red clover*, with  $n = 4$  per treatment



**Fig. 2** Evolution of the proportion of N in clover derived from the atmosphere (PNdfa) over time in white and red clover growing in grass-clover leys. 2007 was the first (GC1), 2008 the second (GC2), and 2009 the beginning of the third year of ley growth. Because the PNdfa was not

significantly affected by the treatments (organic and conventional cropping systems with typical fertilization levels, unfertilized control, and mineral fertilized control), average and standard error of the mean over all five treatments are shown, with  $n=4$  per treatment

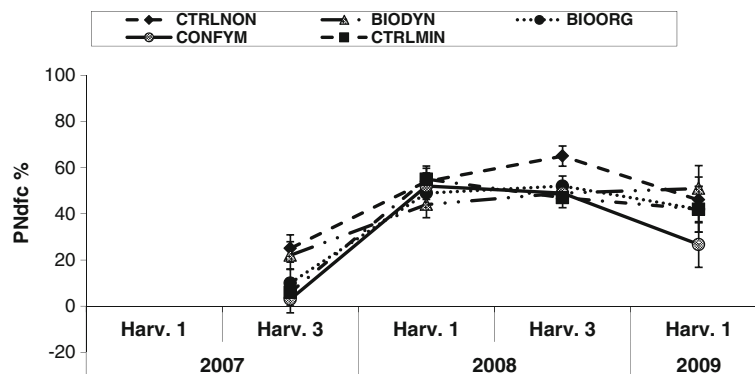
in these treatments, while lowest N:P and N:K ratios suggest that N limited grass yields in CTRLMIN and CONFYM level 2.

For white clover, Mackay et al. (1995) indicated a critical P concentration of  $3.0 \text{ mg g}^{-1}$ , and Whitehead (2000) compiled critical K concentrations from 10 to  $23 \text{ mg g}^{-1}$ . Thus, K clearly limited growth of clover in CTRLNON and probably also in level 1 of BIODYN and BIOORG. Across all treatments, P and K concentrations in ryegrass and both clovers were greater with higher P and K fertilizer inputs, suggesting that co-limitations of P and K may have occurred.

Clover proportions were higher in organic systems (38–53 %) than in conventional systems (25–39 %, Table 5), which resulted in similar clover yields in

organic systems, although total sward yield was lower under organic cropping. These clover proportions in the organic systems were within the very wide range of clover proportions (5–80 %) reported from grass-clover mixtures in an organic cropping field experiment in Denmark (Vinther and Jensen 2000). In an earlier study in the DOK field experiment, clover proportion was not significantly affected by treatments and also varied broadly (6 to 96 %) (Besson et al. 1992).

Lower clover proportions in CTRLMIN and CONFYM than that of organic systems may largely be explained by mineral N inputs, as shown by a significant negative correlation between these two characteristics (Table 8). As shown in many studies, in mixed grass-clover swards a higher mineral N



**Fig. 3** Evolution of the proportion of N in the grass biomass derived from clover in grass-clover leys under organic (BIODYN, BIOORG) and conventional (CONFYM) cropping systems with typical fertilizer input levels, and in the

unfertilized control (CTRLNON) and the mineral fertilized control (CTRLMIN). 2007 was the first (GC1), 2008 the second (GC2) and 2009 the beginning of the third year of ley growth. Average and standard error of mean, with  $n=4$  per treatment

**Table 8** Pearson correlation coefficients for relation between components of annual dry matter yield, symbiotic fixation, mineral N input, and soil and plant nutrient status

	TotalYld	GrassYld	CloverYld	ClovProp	RePNdfa	ANdfa	MinN	SoilP	SoilK	RcNconc	RePconc	RcKconc	GrNconc	GrPconc
GrassYld	0.741***													
ClovYld	0.355 n.s.	-0.363 n.s.												
ClovPrp	-0.287 n.s.	-0.848***	0.786***											
PNdfa	-0.403 n.s.	-0.066 n.s.	-0.485 n.s.	-0.240 n.s.										
ANdfa	0.296 n.s.	-0.407 n.s.	0.973***	0.798***	-0.355 n.s.									
MinN	0.648**	0.826***	-0.251 n.s.	-0.662**	-0.164 n.s.	-0.313 n.s.								
SoilP	0.656**	0.623*	0.058 n.s.	-0.367 n.s.	-0.202 n.s.	-0.047 n.s.	0.626*							
SoilK	0.553 n.s.	0.481 n.s.	0.120 n.s.	-0.249 n.s.	-0.235 n.s.	-0.001 n.s.	0.438 n.s.	0.854***						
RcNconc	0.179 n.s.	0.176 n.s.	0.017 n.s.	-0.076 n.s.	-0.112 n.s.	0.107 n.s.	0.223 n.s.	0.060 n.s.	-0.054 n.s.					
RePconc	0.642**	0.647**	0.007 n.s.	-0.437 n.s.	-0.222 n.s.	-0.076 n.s.	0.620*	0.712***	0.629*	0.154 n.s.				
RcKconc	0.671**	0.852***	-0.246 n.s.	-0.676**	-0.095 n.s.	-0.313 n.s.	0.885***	0.700***	0.665**	0.227 n.s.	0.641**			
GrNconc	0.418 n.s.	0.103 n.s.	0.447 n.s.	0.209 n.s.	-0.572 <sup>x</sup>	0.352 n.s.	0.309 n.s.	0.303 n.s.	0.424 n.s.	0.018 n.s.	0.232 n.s.	0.293 n.s.		
GrPconc	0.574 <sup>x</sup>	0.456 n.s.	0.183 n.s.	-0.202 n.s.	-0.225 n.s.	0.065 n.s.	0.486 n.s.	0.609*	0.643**	0.003 n.s.	0.705**	0.585*	0.380 n.s.	
GrKconc	0.805***	0.652**	0.220 n.s.	-0.304 n.s.	-0.346 n.s.	0.110 n.s.	0.671**	0.699***	0.759***	0.056 n.s.	0.637**	0.814***	0.533 n.s.	0.786***

<sup>x</sup>, \*, \*\*, \*\*\* significant at  $p < 0.10$ , 0.05, 0.01 and 0.001 probability level, respectively

Data from two-year-old grass clover ley growing in 2007 comprising all eight treatments (n=32)

*TotalYld* annual total dry matter (DM) yield, *GrassYld* annual grass DM yield, *ClovYld* annual clover DM yield, *ClovPrp* clover proportion in total yield, *RcPNdfa* proportion of N in red clover derived from atmosphere (see Table 6); *ANdfa* total amount of N fixed by red and white clover (see Table 6); *MinN* annual mineral N input (see Table 1); *SoilP* and *SoilK* soil available P and K (see Table 2); *RcNconc*, *RcPconc*, *RcKconc* N, P and, K concentration in red clover (see Table 7); *GrNconc*, *GrPconc*, *GrKconc* N, P, and K concentration in ryegrass (see Table 7)



fertilizer supply gives grasses a competitive advantage over clovers, especially white clover, and, thus, leads to a lower clover proportion in the swards and to a reduced clover yield (Boller and Nösberger 1987; Hebeisen et al. 1997; Nyfeler et al. 2009). Highest grass and total DM yields under conventional cropping, thus, resulted from highest P, K, and mineral N supply.

$^{15}\text{N}$  isotope signatures and clover N derived from the atmosphere

The fact that  $\delta^{15}\text{N}$  values of both clover species were always below 0 while the non-fixing reference plant ryegrass always had significantly higher  $\delta^{15}\text{N}$  shows that symbiotic  $\text{N}_2$  fixation was the main source of N for both clover species for the duration of the grass-clover ley phase (Fig. 1). Furthermore, the  $\delta^{15}\text{N}$  values of clover were not affected by the  $\delta^{15}\text{N}$  signatures of the animal manures applied to the leys (Table 3). Likewise, the  $\delta^{15}\text{N}$  values of clover were not affected by the treatment specific differences of  $\delta^{15}\text{N}$  signatures of total soil N (Oberson et al. 2007). In contrast, available mineral N taken up by the ryegrass reflected these differences (Fig. 1), as observed by Oberson et al. (2007) for herbaceous weeds growing in the DOK experiment, and by Senbayram et al. (2008) for wheat receiving either mineral fertilizer or manure N in the long term Broadbalk wheat experiment. The highest  $\delta^{15}\text{N}$  values in animal manure of BIODYN translated to the highest  $\delta^{15}\text{N}$  values in ryegrass growing in BIODYN plots, while the lowest  $\delta^{15}\text{N}$  of mineral fertilizers resulted in the lowest  $\delta^{15}\text{N}$  in ryegrass growing in CTRLMIN plots (Table 3, Fig. 1). Because the grass-clover leys received fertilizer inputs (Table 3), the  $\delta^{15}\text{N}$  of plant available mineral N in the soil is affected by the signatures of the two mineral N sources: mineralized soil N and fertilizer N. Thus, use of the reference plant approach is necessary to estimate the  $\delta^{15}\text{N}$  of plant available mineral N in the soil. In our experiment, the reference plant was growing in the same plot as the clover plant, thus, it was exposed to the same soil and fertilizer conditions as the clover plant. This is the best method for use of the reference plant approach (Unkovich et al. 2008; Unkovich and Pate 2000). The  $\delta^{15}\text{N}$  values of ryegrass also demonstrated the need for treatment-specific reference plants (Oberson et al. 2007). In all but one treatment, ryegrass was above the threshold of 2‰ required to apply the natural abundance method when using an

analytical precision in measurement of  $\delta^{15}\text{N}$  of  $\pm 0.2\text{‰}$  (Unkovich et al. 1994). Specifically, ryegrass was below the threshold of 2‰ in CTRLMIN during the second year of grass-clover ley. However, differences between ryegrass and clover growing in CTRLMIN were always significant and were about 2‰.

The small but significant decrease of PNdfa over the 2.5 years could be explained by improved N availability from clover derived N. The high levels of PNdfa found for white and red clover are comparable to the PNdfa obtained from  $^{15}\text{N}$  enrichment studies of grass-clover leys under conventional cropping at moderate N fertilizer inputs (Boller and Nösberger 1987; Nyfeler et al. 2011; Zanetti et al. 1997). Such high levels of PNdfa indicate that growth conditions did not directly limit the process of symbiotic  $\text{N}_2$  fixation (Lüscher et al. 2011). Variation of mineral N input among the treatments from  $6.5 \text{ kg ha}^{-1} \text{ yr}^{-1}$  (BIOORG level 1) to  $150 \text{ kg ha}^{-1} \text{ yr}^{-1}$  (CTRLMIN) (Table 3) did not affect PNdfa. This is in accordance with Nyfeler et al. (2011), who found only a minor effect from increased mineral N fertilizer input from 50 to  $150 \text{ kg N yr}^{-1}$  on PNdfa. The control of symbiotic  $\text{N}_2$ -fixation in ecosystems operates through a series of ecophysiological triggers, and the activity of symbiotic  $\text{N}_2$  fixation is tightly coupled to the gap between N demand (sink) and N availability (source) from mineral N-sources at several scales, from the plant physiology- to the whole ecosystem-scale (Hartwig 1998; Soussana and Hartwig 1996; Soussana et al. 2002). In mixed grass-clover swards, the grass component plays an important role in shaping PNdfa of the clover. Due to the highly competitive ability of grasses to take up mineral N, the mineral N availability remains low for clovers and, thus, PNdfa remains high as long as fertilizer input is only moderately increased (Boller and Nösberger 1987; Nyfeler et al. 2011), and as long as the grass proportion in the sward remains high enough to act as an important sink for mineral N (at least about 40–50 % in the study of Nyfeler et al. (2011)). In the present study, proportion and yield of clover in mixed swards (discussed above) seemed to respond more sensitively to an increased N fertilization than PNdfa did. Furthermore, the strong variation of inputs of P and K, both over the long term (Table 1) and the short term (inputs to the leys, Table 3), that affected the P and K concentrations in clover biomass, did not affect the process of symbiotic  $\text{N}_2$  fixation. The high PNdfa and N concentrations in white and red clover suggest that clover plants in all treatments were able to cover their

internal requirements of N for growth through symbiotic N<sub>2</sub> fixation (Hartwig 1998; Lüscher et al. 2011).

Because PNdfa and N concentration in clover biomass reached the same high level in all treatments ANdfa mainly depended on clover yield of the respective treatment, as earlier reported by Carlsson and Huss-Danell (2003) and Lüscher et al. (2011). Clover yield was strongly inhibited under low P and K availability in CTRLNON and tended to be lower with level 1 fertilization in organic systems, while all other treatments resulted in similar ANdfa. The estimated annual N input by symbiotic fixation of aboveground legume biomass was in the range of ANdfa reported in the literature (Boller and Nösberger 1987; Carlsson and Huss-Danell 2003; Ledgard et al. 2009; Peyraud et al. 2009). The ANdf was also in the same order as annual N inputs by fertilizers (Table 1). However, these estimates do not account for belowground N inputs through rhizodeposition and roots, which may encompass 14 to 74 % of total legume N (Wichern et al. 2008).

#### Grass N derived from clover

The decrease in  $\delta^{15}\text{N}$  in ryegrass over time suggests that the soil N pool that it was exploiting became increasingly affected by clover derived N. In our study, we compared these decreasing values with the  $\delta^{15}\text{N}$  of the ryegrass growing at the start of the grass-clover ley phase, assuming that at this early time it was still unaffected by clover N. This approach is only valid to quantify N transfer from clover to grass if the decrease in  $\delta^{15}\text{N}$  is related solely to clover N and not due to other N sources, such as change in amount or  $\delta^{15}\text{N}$  signature of the fertilizer applied. In this study, there are strong arguments that this assumption is valid: (i) the timing, form, and amount of fertilizer applied was kept constant over the duration of the experiment; (ii) the source of all the fertilizers applied in the different treatments (organic manure, organic slurry, conventional manure, conventional slurry, and synthetic fertilizer) was kept constant throughout the experiment; (iii) the decrease in  $\delta^{15}\text{N}$  occurred in all fertilized treatments and, thus, it is highly improbable that the fertilizer  $\delta^{15}\text{N}$  signature changed in parallel in all the fertilizer sources, which differed among the treatments; (iv) the decrease also occurred in the same range in the unfertilized control, where artefacts through fertilizers cannot occur; and (v) the  $\delta^{15}\text{N}$  signature of *Lolium perenne* in 2008 (3.5‰ and 2.7‰

for BIODYN and BIOORG, respectively, Fig. 1) reached values that were below the soil and fertilizer N sources of the organic treatments (soil total N of 7.6‰ and 7.4‰ for BIODYN and BIOORG, respectively, (Oberson et al. 2007); slurries and manure from 10‰ to 15‰, Table 3) and, thus, must be heavily influenced by clover derived N (−0.6‰, Fig. 1). Alternatively, grass monocultures were used to determine changes in the isotopic composition of available soil N to quantify N transfer (Boller and Nösberger 1987; Høgh-Jensen and Schjoerring 1994; Nyfeler et al. 2011; Zanetti et al. 1997). In the present study, use of this procedure would have required interventions, such as the installation of microplots with grass monocultures and, to maintain identical growth conditions, microplots with grass-clover leys. Microplots are difficult to accommodate in long term field trials and affect plant growth (Oberson et al. 2007). Also the grass monoculture procedure would assume identical soil N dynamics under grass monocultures and grass-legume mixtures for the duration of the experiment, which is questionable because plant species affect soil N dynamics through the rhizodeposition of C and N (Rasmussen et al. 2007).

As most of the aboveground ley biomass (except stolons and stubbles) was removed during the harvests, the source of N must largely have been belowground clover N. Dead clover roots and nodules have been shown to be the most important source of belowground legume N input and transfer to non-fixing plants (Dubach and Russelle 1994; Russelle et al. 1994; Trannin et al. 2000). Death and decomposition of legume roots have been shown to be stimulated by cutting of aboveground biomass (Sierra et al. 2007; Trannin et al. 2000), but the pronounced decrease in  $\delta^{15}\text{N}$  in ryegrass over the winter 2007–2008 suggests significant death and turnover of roots and nodules induced by freezing-thawing. In our study, we used the  $\delta^{15}\text{N}$  of the clover shoot biomass as the source signature for transferred N. Preliminary measurements done on clover roots sampled in spring 2009 resulted in a  $\delta^{15}\text{N}$  of 1.0‰ ( $n=45$ ,  $SD=0.85$ ‰), which is higher than above ground legume biomass. Higher  $^{15}\text{N}$  abundances in legume roots than that in shoots, because of  $^{15}\text{N}$  fractionation, have been reported by Oberson et al. (2007) and suggest that we may have underestimated the PNdfc. Other works have used the  $\delta^{15}\text{N}$  of fixed N (Sierra et al. 2007; Snoeck et al. 2000) or of root exudates as source signatures (Daudin and

Sierra 2008). Our estimates suggest that 46 to 60 % of N taken up by the ryegrass growing in the two-year-old ley was clover derived N. Similar proportions of clover derived N in associated grasses were found by Rasmussen et al. (2007) and Gylfadottir et al. (2007) (40 % and 50 %, respectively) using leaf labeling techniques. As PNdfc was not significantly affected by treatment, and as clover N in each system was largely derived from the atmosphere, belowground N transfer to the grass constitutes an additional input of atmospheric N. Nitrogen concentrations in ryegrass of all treatments suggest that grasses were in need of N and most likely efficiently took up any available N. This strong competitive ability of grasses for mineral N from the soil stimulated symbiotic N<sub>2</sub> fixation in grass-clover mixtures compared to pure clover stands (Nyfeler et al. 2011). Likewise, amount of N transferred from clover to grasses seemed to be related to the N accumulation of the grasses, as also reported by Pirhofer-Walzl et al. (2012).

Overall, amounts of symbiotically fixed N in the aboveground clover biomass per year (60 to 160 kgN ha<sup>-1</sup>yr<sup>-1</sup>) and estimates of atmospheric N in clover below ground N transferred to the grass total an annual atmospheric N input of 90 to 230 kgNha<sup>-1</sup>, which significantly contributes to filling the N gap in the simple N input–output balance presented in Table 1. These amounts do not yet comprise clover N derived from the atmosphere which has been incorporated into microbial biomass and soil organic matter (Mayer et al. 2003).

## Conclusions

Proportion of N in clover derived from the atmosphere was very high in all cropping systems and fertilizer levels, even under the strongly nutrient-scarce unfertilized control. Thus, it is evident that cropping systems and fertilization level did not directly limit the process of symbiotic N<sub>2</sub> fixation in clover. Higher clover proportions in organic compared to conventional cropping resulted in comparable clover yields and ANdfa in these systems, although total sward yield was lower under organic cropping. Changes in the <sup>15</sup>N isotopic signature over time suggest significant clover N transfer to the associated grasses. In this long-term experiment, both organically and conventionally cropped grass-clover leys fully profited from symbiotic N<sub>2</sub>

fixation, provided sufficient P and K supply to sustain clover biomass production.

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